

The Kinetics of Appearance of Chloroplast Proteins and the Effect of Cycloheximide and Chloramphenicol on Their Synthesis

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Proplastids in dark grown *Euglena gracilis* cells undergo a developmental process during illumination yielding full functional chloroplasts. The appearance of chlorophyll, cytochrome 552, cytochrome 563, and ribulose-1,5-bisphosphate carboxylase in these cells was investigated and different kinetics of synthesis were observed for each component.

Light adaptation experiments on dark grown cells in the presence of the 70S ribosome inhibitor, chloramphenicol, revealed that the synthesis of cytochrome 552 was blocked but not cytochrome 563. In presence of the 80S ribosome inhibitor cycloheximide, the synthesis of both cytochromes as well as of chlorophyll (a+b) and of ribulose-1,5-bisphosphate carboxylase were inhibited. However, at low concentrations of cycloheximide (3.5 to 9×10^{-6} M), a stimulation of the synthesis per cell of the cytochromes, of chlorophyll (a+b) and of ribulose-1,5-bisphosphate carboxylase was observed.

Introduction

Euglena gracilis is an useful organism for studying the greening process in plastids, because the cells can be grown under heterotrophic conditions where they contain no chlorophyll and upon illumination a transversion of proplastids to fully functional chloroplasts occurs^{1–3}.

The discovery of DNA, ribosomes, enzymes, and factors necessary for the transcriptional and translational processes in chloroplasts promoted the idea of their autonomous reproduction in the cell. Further investigation indicated rather the dependence of the plastid on the rest of the cell. This dependence can be studied very closely during the developmental process^{4–6}.

Our studies report on the kinetics of the appearance of some characteristic plastid proteins and chlorophyll during the greening process. The influence of D(-)threo-chloramphenicol and cycloheximide in wide ranges of concentrations on cell growth and on the synthesis of chlorophyll (a+b), ribulose-1,5-bisphosphate carboxylase and of cytochrome 552 and cytochrome 563 was also investigated.

Materials and Methods

Cultures of *Euglena gracilis*, strain Z (Pringsheim) were grown at 22 °C under photoautotrophic, mixotrophic, or heterotrophic conditions. The growth medium of Cramer and Myers⁷ modified by Böger and San Pietro⁸ was supplemented with glutamate 1.5 g, sucrose 7.5 g, aspartate 1 g, malate 0.5 g, glycine 1.25 g and succinate 0.23 g per liter as carbon sources for the mixotrophic and heterotrophic culture medium. The pH was adjusted to 4.0. The cell cultures were gassed with 5% (v/v) CO₂ in air and illuminated with white light (1500 lx).

The greening experiments were carried out with dark grown cell cultures in the logarithmic growth phase (cell density 2×10^6 cells per ml). The cultures were diluted with fresh medium to a cell density of $0.1 - 0.5 \times 10^6$ cells per ml. Additions (cycloheximide, chloramphenicol) were made as indicated in the Results and the cultures were kept as mentioned above.

Cell counting was performed by the use of a counting chamber, chlorophyll was determined by the method of Arnon⁹.

The ribulose-1,5-bisphosphate carboxylase activity assay was based on the fixation of ¹⁴CO₂ into acid-stable products by a cell sonicate with ribulose-1,5-bisphosphate (RuDP) and H¹⁴CO₃⁻ as substrates¹⁰. The cells were harvested from the culture and washed with 100 mM Tris-buffer (pH 8.0) containing 10 mM MgCl₂. The cells were suspended in

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the same buffer and sonicated with a Branson sonifier model S75 in order to break all of the cells. Assay mixtures contained in 200 μ l: 40 μ l of cell sonicate, 0.5 μ mol RuDP, 5 μ mol $\text{H}^{14}\text{CO}_3^-$ (1 mCi/ μ mol) and 0.6 μ mol MgCl_2 in 0.1 M Tris-buffer (pH 8.0).

The reactions were carried out immediately after sonification at 25 °C. After 10 min the assay reactions were stopped by addition of 50 μ l glacial acetic acid; aliquots were spotted onto paper strips and the amount of fixed ^{14}C on the dried strips was determined by liquid scintillation counting.

The capability of whole cells to fix $^{14}\text{CO}_2$ was estimated with a cell sample suspended in 10 mM Tris-buffer (pH 8.0) containing 10 mM $\text{H}^{14}\text{CO}_3^-$. The CO_2 -photoassimilation reaction was carried out under saturating light intensities of 40 000 lx at 25 °C. The reaction was stopped by addition of 50 μ l of a mixture of concentrated HCl and glacial acetic acid (4:1) to an aliquot of 100 μ l of the cell suspension.

The contents of cytochrome 552 and 563 in the cell sonicate were estimated from difference spectra recorded with an AMINCO MW-2 spectrophotometer. Cytochrome 552 was determined from the difference in extinction at 552–540 nm of an untreated and a ferricyanide oxidized sample and corrected for any nonlinearity in the baseline¹¹. Cytochrome 563 content was calculated from the difference in extinction at 563–575 nm of a sample containing ascorbate and another containing dithionite. The base lines were monitored before and after the additions. The slit width was adjusted to 2 nm and the recording speed was 2 nm per second.

Results

The induction of carbon dioxide fixation in greening cell cultures

The results of a typical greening experiment are summarized in Fig. 1 for a heterotrophically grown culture which was kept for 30 generations in the dark and then illuminated with white light of 1500 lx. The culture in the logarithmic growth phase was gassed with 5% CO_2 in air and kept at 22 °C. Chlorophyll synthesis starts after a lag phase and continues linearly during the course of development. A constant level was reached at 4 mg chlorophyll (a+b) per 10^8 cells. Dark grown cells have almost no RuDP carboxylase activity. After illumination, however, there is a fast rise in the production of this enzyme.

After 20 hours of illumination, a plateau of enzyme activity per cell was observed at 40 μ mol of

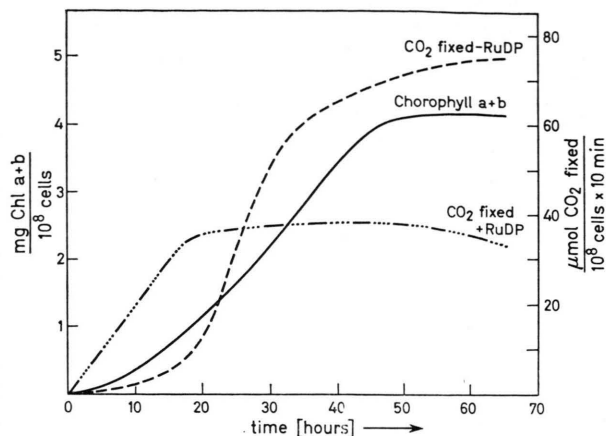


Fig. 1. The induction of carbon dioxide fixation in greening cell cultures. The appearance of ribulose-1,5-bisphosphate carboxylase activity (— · — · — · —), of chlorophyll (a+b) (—), and the measurement of the CO_2 fixation in intact cells (---) during the greening process. Details of the assays are given in the Methods.

CO_2 fixed per 10^8 cells and per 10 min. The changing rate of photosynthesis during chloroplast development was also monitored by addition of $\text{H}^{14}\text{CO}_3^-$ to a sample of whole cells illuminated with saturating light intensities. The optimal photosynthetic rate was reached after 72 hours of illumination and calculated to be 120 μ mol CO_2 fixed per mg chlorophyll and per hour.

Light induced biosynthesis of chloroplast cytochromes

Fig. 2 shows the kinetics of appearance of cytochrome 552 and cytochrome 563 in a greening experiment. After the onset of illumination, a linear increase of both cytochromes is observed. The different slopes of the curves indicate that the cytochromes are synthesized at different rates. In the first hours of the greening process more cytochrome 563 is synthesized in comparison to cytochrome 552. Using a millimolar extinction coefficient of 20 for the reduced minus oxidized forms of the cytochromes, the ratio of chlorophyll to cytochrome 563 varied between 100 (at 40 hours) to 200 (at 68 hours), and of chlorophyll to cytochrome 552 between the values 330 and 250 at 40 and 68 hours respectively.

Inhibition of cytochrome biosynthesis by antibiotics

The influence of the antibiotics D(–)-threo-chloramphenicol and cycloheximide on plastid develop-

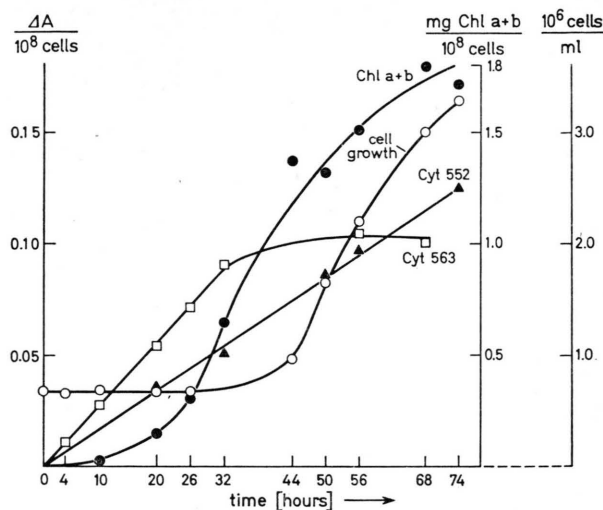


Fig. 2. The induction of cytochrome synthesis in greening cell cultures. The appearance of cytochrome 552 (▲-▲) and of cytochrome 563 (□-□) in comparison to cell growth (○-○) and chlorophyll (a+b) synthesis (●-●).

ment and cytochrome biosynthesis were investigated to elucidate the sites of translation. Increasing amounts of the antibiotics were supplemented to dark grown cultures and after 72 hours of illumination under mixotrophic conditions the cells were harvested and examined. The results of the experiments with chloramphenicol are summarized in Figs 3 and 4. Concentration as high as 1 mg per ml cell culture had no influence on cell division but the chlorophyll content of the cells was reduced to one-half and the RuDP carboxylase activity dropped to one-third. Fig. 4 shows the cytochrome content of chloramphenicol treated cells. The amount of cytochrome 552 per cell decreases at higher chlor-

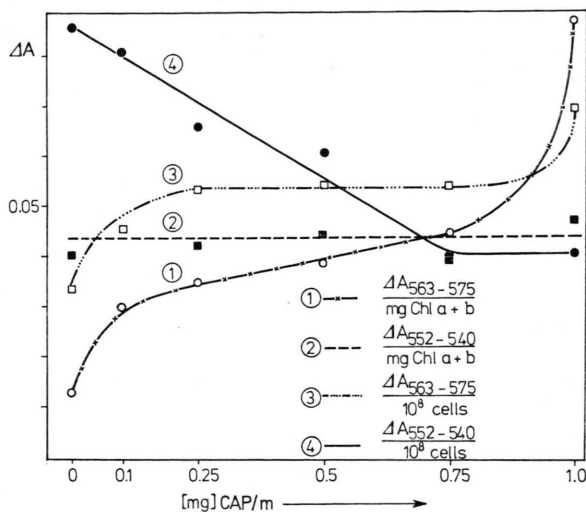


Fig. 4. The influence of chloramphenicol on cytochrome synthesis. The content of the cells on cytochrome 563 (on chlorophyll basis - curve 1, on cell basis - curve 3) and on cytochrome 552 (on chlorophyll basis - curve 2, on cell basis - curve 4) in dependence of the chloramphenicol concentration in the culture medium after 72 hours of illumination.

amphenicol concentrations, but remains constant on a chlorophyll basis. In contrast, there is almost no influence of chloramphenicol on the synthesis of cytochrome 563, which results in an increased ratio of cytochrome 563 to chlorophyll.

The same studies were carried out with cycloheximide in a concentration range from 1 to 15 μ g per ml cell culture. A heterotrophically grown inoculum in the logarithmic growth phase was diluted with fresh medium and increasing amounts of cycloheximide were added to different batches of the culture. Immediately after the introduction of the

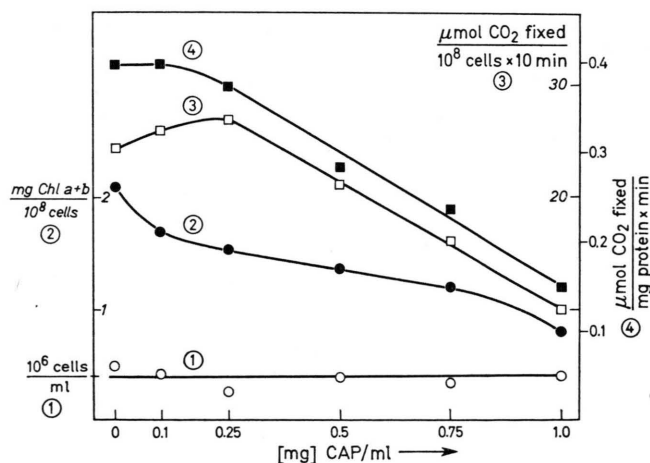


Fig. 3. The influence of chloramphenicol on the greening process. The cell growth (curve 1), the chlorophyll (a+b) content of the cells (curve 2), their ribulose-1,5-bisphosphate carboxylase activity (curve 4) and their photosynthetic carbon dioxide fixation capability (curve 3) in dependence of the chloramphenicol concentration in the culture medium after 72 hours of illumination.

antibiotic to the cell cultures, the light was turned on and the cell were aerated. After 72 hours of illumination the cell samples were analyzed. Fig. 5 shows the inhibition of cell growth (curve 1) due to cycloheximide. Low concentrations of the antibiotic

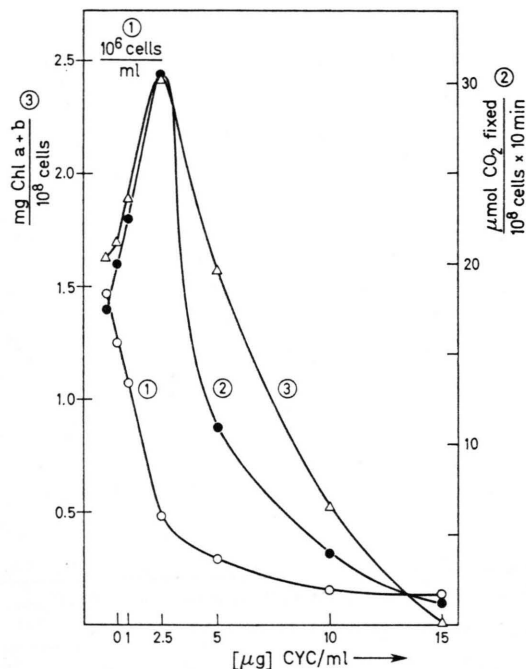


Fig. 5. The inhibition of the greening cells by cycloheximide. Cell growth (curve 1), chlorophyll (a+b) synthesis (curve 2) and ribulose-1,5-bisphosphate carboxylase activity (curve 3) in dependence of the cycloheximide concentration in the culture medium after 72 hours of illumination.

caused a drastic reduction of cell division and concentration above 10 µg a complete stop of cell multiplication. Chlorophyll synthesis (curve 2) was stimulated at low concentrations but fully inhibited at 15 µg cycloheximide per ml cell culture. Curve 3 is similar in shape to curve 2 and represents the activity of RuDP carboxylase in the cells.

The results of the studies concerning the relationship between cytochrome synthesis and cycloheximide concentrations are summarized in Fig. 6. The synthesis of both cytochromes is strongly inhibited at high concentrations of the antibiotic. A stimulatory effect at low concentrations of cycloheximide is more pronounced in the case of cytochrome 552 on a cell basis, however, on a chlorophyll basis only inhibition is observed indicating that chlorophyll itself shows the highest transient stimulation.

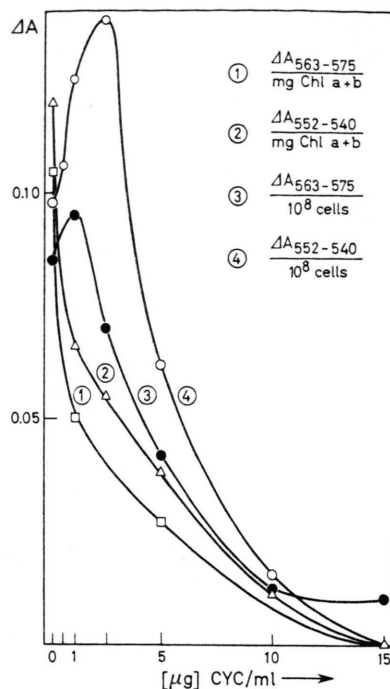


Fig. 6. The inhibition of cytochrome synthesis by cycloheximide. The content of the cells on cytochrome 563 (on chlorophyll basis — curve 1, on cell basis — curve 3) and on cytochrome 552 (on chlorophyll basis — curve 2, on cell basis — curve 4) in dependence of the cycloheximide concentration in the culture medium after 72 hours of illumination.

Discussion

When dark adapted *Euglena gracilis* cells are illuminated, the synthesis of chlorophyll, RuDP carboxylase, cytochrome 552, and cytochrome 563 begins immediately (Figs 1 and 2). The rate of synthesis of these chloroplast components are, however, quite different and the plateau levels (the level of constant ratio on a cell basis) are reached at different times. In the first 40 hours of chloroplast development no cell multiplication occurs even though a non-synchronized cell culture was used for these experiments. The energy of cell metabolism seems to be diverted to cell adaptation rather than to cell growth in order to meet the requirements for the new environmental conditions.

The significant differences in the kinetics of synthesis of cytochrome 552 and cytochrome 563 may also reflect the fact that cytochrome 552 is only weakly bound to the thylakoids in *Euglena* chloroplasts whereas cytochrome 563 is rather firmly

bound and therefore more important for the structural organization of the membrane¹¹.

Interesting was the observation that the assimilation curve for carbon dioxide crosses the RuDP carboxylase activity curve after 25 hours of illumination (Fig. 1). It appears that the enzyme in the crude cell sonicate is less active than *in vivo*. Preliminary experiments concerning the kinetic properties of RuDP carboxylase from *Euglena gracilis* indicate that the V_{\max} value of the enzyme is lower immediately after cell rupture than after 3 hours. This effect can be explained as metabolic inhibition (e.g. 6-phosphogluconate) of the enzyme in the crude sonicate. After a longer storage time, these compounds are further metabolized and the enzyme activity reaches its maximal value.

The translational studies suggest that cytochrome 563 is synthesized on 80S ribosomes whereas cytochrome 552 needs both 80S and 70S ribosomes. The observation that this small cytochrome molecule (m.w. 12 000 daltons) needs two different translational sites can be explained by a mechanism proposed by Kadenbach¹² for cytochrome c from animal tissue. In this system, the apoprotein of the mitochondrial cytochrome is synthesized on the ribosomes in the cytoplasm but the binding step of the heme to the protein moiety is catalysed by a protein which is produced on the 70S ribosomes. Such a mechanism might also be operating for *Euglena* and cytochrome 552.

Contrary to the results presented in this paper, Smillie and co-workers¹³ reported that, in *Euglena gracilis*, a b-type cytochrome 561 was also inhibited by chloramphenicol and furthermore that cycloheximide had no influence on the synthesis of RuDP carboxylase. Under our conditions the synthesis of RuDP carboxylase was inhibited by both antibiotics suggesting that 70S and 80S ribosomes are involved in enzyme production. Criddle *et al.*¹⁴ have been able to show that the large subunit of the

enzyme is synthesized on 70S ribosomes in chloroplasts of barley leaves and that the small subunit is synthesized on cytoplasmic ribosomes. These results were confirmed by Wildman and Kawashima¹⁵ using a genetic approach, recently by Schiff and co-workers¹⁶ and by Davis and Merrett¹⁷ in *Euglena gracilis*.

Another interesting aspect concerning the synthesis of the chloroplast proteins arises from the experiments with cycloheximide at low concentrations. An explanation for the stimulatory effect of the antibiotic on protein synthesis in the range of 3.5 to 9×10^{-6} M can only be made on a speculative basis. It might be possible that a negative control mechanism, such as the synthesis of a nuclear gene repressor, is more sensitive to the action of cycloheximide than the translation of the chloroplast proteins which are synthesized on the cytoplasmic ribosomes. Ohad and Drews⁶ proposed a hypothesis on the regulation of chloroplast development; the scheme includes the participation of cytoplasm and chloroplasts on the formation of photosynthetic membranes and its control mechanism is exerted by an active repressor on the transcription of nuclear DNA. In this connection it should be mentioned that a chloroplastless mutant, which was derived from high temperature treatment of *Euglena* cells, is not able to synthesize cytochrome 563 in the light despite the fact that this cytochrome is coded by a nuclear gene. Thus, the start of the greening process after the onset of illumination may involve a signal from the plastids to the nucleus to produce m-RNA's for the synthesis of plastid proteins.

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